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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE RELATIONSHIP OF BLOOD-GROUPS TO DISEASE.*

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Forwarded for publication August 16th, 1921.

THE possibility of a fixed relationship of "blood-group" to a particular disease or diseases has received practically no consideration. A fundamental property of blood was recognised first by Landois Eulenburg (1866), when he demonstrated that the serum of one animal may have the property of destroying the red cells of another animal of the same species. This property is now known as isohæmolysis, and is of prime importance in the therapeutic application of our knowledge of blood grouping. On isohæmolysis certain poor results of transfusion depend; thus it is necessary to make a proper blood-grouping.

The salient feature on which the actual grouping of blood rests was discovered by Ehrlich (1900). He observed the agglutination of erythrocytes by serum from animals of the same species. Ehrlich called this property iso-agglutination. At a much earlier period Jones (1853) and, later, Paget (1871) observed a peculiar tendency of blood from persons with certain acute infections to undergo a rapid agglutination (rouleau formation) when poured on a glass plate. Shattock (1900), stimulated by the work of Landois and

* This investigation was made in the Department of Clinical Laboratories of the Mayo Clinic under the direction of Dr. A. H. Sanford.

Ehrlich, and interested by the observations of Jones and Paget, was the first to observe iso-agglutination in man. He mixed the serum of a patient with pneumonia with the erythrocytes of a normal person. He observed prompt agglutination of the red cells. He later made the same test with the serum of a patient with acute rheumatism and the erythrocytes of a patient with leukæmia. The same phenomenon occurred. He watched the rheumatic patient through convalescence; the peculiarity persisted.

Landsteiner (1901), influenced by the work of Shattock, began matching the blood of healthy persons. He was able to classify all persons, because of the properties of their blood, into three groups. The groups described by Landsteiner are now known as Groups II, III and IV. In a later study Jansky (1907) discovered a fourth group, now known as Group I. The same observation was made independently by Moss (1909). A difference in their groupings was that Jansky considered Group I the common group and Group IV the uncommon group. Moss classified his groups in the reverse order. At the Mayo Clinic the Moss classification is used, although the priority of Jansky is recognised.

Hektoen (1907) demonstrated that disease fails to modify iso-agglutination in man. Bond (1920) has recently called attention to the frequency of auto-agglutination in the presence of a positive Wassermann reaction, focal infection, pneumonia, empyema, typhoid fever, carcinoma of the uterus with secondary infection, and in a number of cases of infective endocarditis. Such phenomena have not been observed in the Mayo Clinic.

The Mendelian transmission with regard to blood-grouping was studied by von Dungern and Hirschfeld (1911), who concluded that the groups were transmissible according to the Mendelian laws. Their study consisted of seventy-one families, and their investigations were carried through only two generations. Ottenberg and Epstein (1908) also presented data in accord with the findings of von Dungern and Hirschfeld. This phase of the problem needs further investigation, because transmission of more than one character requires study through more than two generations to prove the fulfilment of the Mendelian laws. This fact becomes evident when families are grouped for the purpose of securing a donor, and often groups are isolated that are not represented in either parent.

The data of the Mendelian transmission were accepted by Alexander (1921), who was led to make a series of studies on certain patients in order to find if there is linkage with a factor on which certain blood diseases depend. He studied particularly malignancy, and concluded "that while persons belonging to all four groups are liable to malignant disease, those in Groups I and III appear to be peculiarly susceptible, and the clinical type of disease is generally speaking more malignant." Alexander failed to consider the influence of nationality in his series.

The data presented in our study is a review of the blood-groups found in patients in the Mayo Clinic from January 1st, 1917, to May 1st, 1921. The relationship between blood-grouping and disease is shown in Tables 1 to 17, but only when a considerable number of cases were available have the figures been carried out into percentages. Percentages based on a small number of cases are quite often misleading.

For the sake of comparison the original percentages of Moss and of Hirschfeld and Hirschfeld (1919) on nationality and blood-grouping are discussed.

Original Percentages of Moss.

Group I	10	per cent.
„ II	40	„
„ III	7	„
„ IV	43	„

Abbreviated Table of Hirschfeld and Hirschfeld on Blood-grouping and Nationality.

	Group I per cent.	Group II per cent.	Group III per cent.	Group IV per cent.
English	3·0	43·4	7·2	46·4
French	3·0	42·6	11·2	43·2
Italians	3·8	38·0	11·0	47·2
Germans	5·0	43·0	12·0	40·0
Austrians	8·0	40·0	10·0	42·0
Serbs	4·6	41·8	15·6	38·0
Greeks	4·0	41·6	16·2	38·2
Bulgarians	6·2	40·6	14·2	39·0
Arabs	5·0	32·4	19·0	43·6
Turks	6·6	38·0	18·6	36·8
Russians	6·3	31·2	21·8	40·7

TABLE 1.—292 *Cases of Carcinoma.*

	Group I.	Group II.	Group III.	Group IV.
United States	6	87	15	99
Russia	1	5	—	1
Scandinavia	2	11	4	10
Germany	2	11	1	11
England	—	2	1	16
Bohemia	—	2	—	2
Poland	—	1	—	—
Austria	—	—	1	—
France	—	—	—	1
	11 (3·76 %)	119 (40·75 %)	22 (7·53 %)	140 (47·94 %)

The data in Table 1 contain nothing to show a relationship of the factor or factors responsible for the blood-group to carcinoma. The percentages for Groups I and III are entirely out of accord with those of Alexander.

TABLE 2.—457 *Cases of Pernicious Anæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	17	147	31	144
England	1	16	6	21
Russia	1	3	—	1
Scandinavia	1	19	6	17
Germany	—	15	3	4
Belgium	—	2	—	1
Italy	—	—	—	1
	20 (4·37 %)	202 (44·20 %)	46 (10·06 %)	189 (41·35 %)

The percentages in Table 2, although at slight variance with the original percentages of Moss, demonstrate that the causal factor in pernicious anæmia has no relation to blood-grouping.

TABLE 3.—11 *Cases of Acute Lymphatic Leukæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	3	3	4
Germany	—	—	—	1
	—	3	3	5

TABLE 4.—16 *Cases of Chronic Lymphatic Leukæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	3	—	9
England	—	1	—	—
Scandinavia	—	1	—	—
Russia	—	—	1	—
Germany	—	—	1	—
	—	5	2	9

TABLE 5.—47 *Cases of Myelogenous Leukæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	1	12	6	15
England	—	2	2	3
Russia	—	—	1	—
Scandinavia	—	—	2	1
Germany	—	—	—	2
	1	14	11	21

TABLE 6.—41 *Cases of Splenic Anæmia.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	14	1	19
Scandinavia	—	1	—	—
Russia	—	1	—	1
Austria	—	1	—	—
England	—	—	—	2
Greece	—	—	—	1
	—	17	1	23

TABLE 7.—20 *Cases of Hæmophilia and* 20 *Cases of Purpura.*

	Group I.	Group II.	Group III.	Group IV.
United States	1	13	3	18
Russia	—	3	1	—
Scandinavia	—	—	—	1
	1	16	4	19

TABLE 8.—62 *Cases of Acute and Chronic Kidney Disease.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	17	3	22
England	—	2	1	7
Austria	—	2	—	—
France	—	—	—	1
Germany	—	—	—	3
Poland	—	—	—	1
Scandinavia	—	—	—	3
	—	21	4	37

TABLE 9.—20 *Cases of Cardiac, Valvular and Myocardial Disease.*

	Group I.	Group II.	Group III.	Group IV.
United States	—	6	2	10
England	—	1	—	—
Scandinavia	—	—	1	—
	—	7	3	10

TABLE 10.—37 *Cases of Diseases of the Thyroid.* (15 *Cases of Exophthalmic Goitre,* 19 *Cases of Adenoma without Hyperthyroidism,* 2 *Cases of Myxœdema, and* 1 *Case of Colloid Goitre.*)

	Group I.	Group II.	Group III.	Group IV.
United States	—	9	1	19
England	—	2	1	2
Scandinavia	—	1	—	1
Germany	—	—	—	1
	—	12	2	23

TABLE 11.—65 *Cases of Tumours, Benign and Malignant, other than Cancer.*

	Group I.	Group II.	Group III.	Group IV.
United States	1	15	6	22
Italy	—	1	—	2
England	—	1	—	3
Scandinavia	—	3	1	2
Germany	—	1	1	3
Austria	—	—	2	1
	1	21	10	33

The data in Tables 3 to 11, although small, are sufficient to justify the suspicion that there is no linkage.

TABLE 12.—134 *Cases of Calculi.* (130 *Cases of Cholelithiasis*, 2 *Cases of Nephrolithiasis, and* 2 *Cases of Vesicolithiasis.*)

	Group I.	Group II.	Group III.	Group IV.
United States	4	39	9	48
Scandinavia	—	8	1	—
England	—	2	—	10
Germany	—	4	1	1
Bohemia	—	1	—	1
Russia	—	1	1	1
Switzerland	—	—	—	1
France	—	—	—	1
	4 (2·97 %)	55 (41·04 %)	12 (8·95 %)	63 (47·01 %)

TABLE 13.—137 *Cases of Fibroid Uterus.*

	Group I.	Group II.	Group III.	Group IV.
United States	3	52	11	45
Scandinavia	3	2	—	2
England	—	7	2	5
Switzerland	—	1	—	—
Germany	—	2	—	1
Belgium	—	—	—	1
	6 (4·37 %)	64 (46·71 %)	13 (9·48 %)	54 (39·41 %)

TABLE 14.—194 *Cases of Secondary Anæmia from all Causes.*

	Group I.	Group II.	Group III.	Group IV.
United States	8	62	16	56
England	1	10	4	15
Germany	—	4	1	5
Holland	—	1	—	—
Scandinavia	—	2	—	4
Bohemia	—	1	—	—
Russia	—	2	—	1
Austria	—	—	—	1
	9	82	21	82
	(4·63 %)	(42·26 %)	(10·72 %)	(42·26 %)

TABLE 15.—176 *Cases of Acute and Chronic Gall-Bladder Disease.*

	Group I.	Group II.	Group III.	Group IV.
United States	5	41	10	69
Germany	1	4	1	4
Bohemia	—	1	—	1
Austria	—	1	—	—
England	—	7	—	19
Scandinavia	—	6	1	—
Holland	—	1	—	—
Switzerland	—	—	—	1
Russia	—	1	1	1
	6	62	13	95
	(3·40 %)	(35·39 %)	(7·38 %)	(53·97 %)

There is a slight diminution in the percentages for Group II as shown in Table 15 with a considerable increase in Group IV. This, however, can have nothing to do with heredity.

TABLE 16.—172 *Cases of Chronic Ulcers.* (42 *Cases of Gastric Ulcer,* 102 *Cases of Duodenal Ulcer,* 4 *Cases of Gastro-jejunal Ulcer,* 15 *Cases of Chronic Ulcerative Colitis, and* 8 *Miscellaneous Cases.*)

	Group I.	Group II.	Group III.	Group IV.
United States	5	44	3	76
England	2	2	3	15
Scandinavia	—	7	—	5
Germany	—	2	—	3
Austria	—	—	2	—
Bulgaria	—	—	—	1
Russia	—	—	—	1
Honduras	—	—	—	1
	7	55	8	102
	(4·07 %)	(31·97 %)	(4·65 %)	(59·30 %)

TABLE 17.—173 *Cases of Jaundice, all Causes.*

	Group I.	Group II.	Group III.	Group IV.
United States	5	63	12	46
Germany	1	7	2	2
England	—	6	1	14
Italy	—	1	—	—
Scandinavia	—	6	3	—
Belgium	—	—	1	—
Austria	—	—	1	—
Bohemia	—	—	—	1
Switzerland	—	—	—	1
	6 (4·04 %)	83 (47·97 %)	20 (11·56 %)	64 (36·99 %)

TABLE 18.—372 *Miscellaneous Cases.* (*Group of Patients with Various Non-Malignant Diseases.*)

Group I.	Group II.	Group III.	Group IV.
19 (5·10 %)	150 (40·32 %)	26 (6·96 %)	177 (47·58 %)

TABLE 19.—2446 *Cases of Combined Diseases.*

Group I.	Group II.	Group III.	Group IV.
91 (3·31 %)	988 (40·45 %)	221 (9·09 %)	1146 (46·85 %)

The analysis of the blood-groups in 2446 patients (Table 19) gives percentages that are quite different from those originally found by Moss. The variation is explainable by nationality, and in some instances by the small number of patients considered in certain series. The difference is noticeable in all the tabulations, and for that reason it is well to consider the figures of Moss as only approximate, since they vary within certain limits in each group in each series. Sanford (1920), in a statistical study of about 3000 patients and donors, found 4·5 per cent. in Group I, 42·3 per cent. in Group II, 8·7 per cent. in Group III, and 44·5 per cent. in Group IV. Since then Culpepper and Ableson (1921), in an analysis of 5000 blood-groups, found that there were 5·18 per cent. in Group I, 35·06 per cent. in Group II, 14·28 per cent. in Group III, and 44·47 per cent. in Group IV.

CONCLUSIONS.

(1) There is no relationship between blood-groups and malignancy as suggested by Alexander.

(2) There is no relationship between blood-groups and any disease in which sufficient data are available to justify a conclusion.

(3) The percentages originally presented by Moss are approximate and capable of considerable variation, without special significance.

(4) Nationality should be taken into consideration in the presentation of statistical studies of blood-grouping.

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DYSENTERY IMMUNISATION IN RABBITS BY THE ORAL AND SUBCUTANEOUS METHODS.

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THE possibility of immunisation *per os* was first demonstrated by Ehrlich (1891), who succeeded in eliciting the development of anti-ricin in rabbits by feeding them with ricin. This question was left more or less in abeyance until 1905, when Zeitlin (1905) showed that rabbits, to which a killed suspension of *B. dysenteriæ* (Shiga) had been administered orally, developed agglutinins for that organism in their serum. In view of these findings he suggested that the oral route should be employed in the prophylactic immunisation of man against such acute intestinal diseases as cholera, dysentery and enteric. Working along these lines Hida and Toyoda (1907) demonstrated the production of antibodies in guinea-pigs by the administration *per os* of dysentery bacilli (type Shiga) previously digested with pepsin and trypsin. The ingestion of dysentery bacilli, which had merely been killed by heating to 60° C., was, according to these workers, unable to give rise to any antibody response, but Shiga (1908) claims to have immunised rabbits against this same organism by the oral administration of heat-killed vaccines.

More recently Besredka (1919) brings forward experimental evidence in support of oral immunisation. He claims that a solid immunity, as tested by intravenous injection of the live organism, can be produced in rabbits against *B. dysenteriæ* (Shiga), *B. paratyphosus B.* and *B. typhosus* by the introduction *per os* of killed vaccines. Rabbits immunised in this manner were able to support the intravenous inoculation of otherwise lethal doses of living organisms. This method of immunisation was found by him to be more efficacious in the cases of *B. paratyphosus* and *B. typhosus* if the vaccine was mixed with a certain quantity of sterile bile. From his observations Besredka concluded that the intestinal mucosa forms the first line of defence against

invasion by these organisms—or the toxic products of these organisms—which give rise to acute intestinal conditions. This contention is supported by the observation that the first ingestion of antigen gave rise to the appearancé of antibody in the blood, whereas the subsequent ones did not; in fact, at that period where he was able to demonstrate immunity in his animals, the agglutinins had practically disappeared. This immunity he therefore considers to be mainly local—an increased resistance on the part of the cells of the intestinal mucous membrane to the passage of the infecting organism or of its toxin—the damaged cells which are cast off in the catarrhal condition set up by the infection being replaced by new ones possessed of this acquired property. Besredka looks upon this initial catarrhal stage as essential to the establishment of a satisfactory local immunity, and for this reason bile is administered with the vaccine.

In appraising the practical value of any method of prophylactic immunisation we should consider the following points:

(I) The degree of immunity obtained.
(II) The intensity of reaction, local and general, produced by the vaccine.
(III) The simplicity of administration.

It is generally recognised that the local and general reactions following the subcutaneous inoculation of Shiga vaccines in all but low doses are often intense. In view, therefore, of Besredka's finding that a solid immunity may result from the oral administration of the vaccine, it was decided to carry out experiments in rabbits with the object of determining the degree of such resistance to *B. dysenteriæ* (Shiga), and to compare it with the immunity resulting from the usual subcutaneous inoculation.

SCHEME OF RESEARCH.

First Series of Experiments.

Two strains of *B. dysenteriæ* (Shiga) were employed in this investigation. One came from the National Collection of Type Cultures (No. 151), and for the other I am indebted to the kindness of Dr. Besredka. Both these strains were typical, culturally and serologically. The work reported in this communication was carried out with strain No. 151 practically entirely.

Ingestion of vaccines.—The 24-hour growth on agar obtained from one Roux bottle was suspended in 20 c.c. of sterile normal saline and heated for one hour at 60° C. Measured quantities of this suspension were mixed with bran and fed to rabbits which had fasted for 24 hours.

Antibody production (agglutinins).—Test bleedings were made before, and at intervals during the immunisation. The titre of antibody was determined by means of the agglutination reaction, results being read after four hours' incubation at 37° C. and again after standing at room temperature for twenty-four hours.

Degree of immunity produced.—This was determined by the intravenous inoculation of lethal doses of living *B. dysenteriæ* (Shiga), the virulence of which for the rabbit had been previously determined.

EXPERIMENTAL DATA.

The Virulence of Strain No. 151.

A saline suspension of a 24-hour agar slope culture was inoculated intravenously in a series of four rabbits, the dose varying from 50×10^6 to 500×10^6. The results are recorded in Table I:

TABLE I.

No. of rabbit.	Weight.	Number of bacilli inoculated.	Result.
17	1740 grm.	500×10^6	Died 2 days after injection.
18	1535 ,,	250×10^6	,, 8 ,, ,, ,,
19	1700 ,,	100×10^6	Survived.
20	1450 ,,	50×10^6	Survived.

Post-mortem Findings.

No. 17.—Small intestine congested and mucous membrane œdematous. The organism was recovered from the intestinal contents but not from the spleen.

No. 18.—Small intestine showed the same changes as in Rabbit 17. No hæmorrhages. Large intestine and appendix appear normal. Cultures from the heart-blood, gall-bladder, spleen and intestinal contents proved sterile.

From these experiments it would appear that the M.L.D. of this strain was about 500×10^6. In later experiments this dose was sometimes found insufficient to kill medium-sized rabbits, and accordingly 1000×10^6 was adopted as the test dose.

FIRST SERIES OF EXPERIMENTS.

Effect of Oral Immunisation.

Eight rabbits were immunised, as follows:

Nos. 5 and 6 received $\frac{1}{2}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 7 and 8 received $\frac{1}{5}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 9 and 10 received $\frac{1}{10}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 11 and 12 received $\frac{1}{5}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

In the two latter (Nos. 11 and 12) the ingestion was preceded by the oral administration of 8·0 c.c. of ox bile. Of these eight rabbits two died, No. 5 on the fifteenth day after ingestion and No. 8 on the fourteenth day. No obvious cause for death was found. The remaining six rabbits were divided into two groups. Group 1 (Nos. 6, 7 and 9) were inoculated with 500×10^6 living bacilli intravenously on the nineteenth day after the ingestion of the killed dysentery bacilli. Group 2 (Nos. 10, 11 and 12) received 1000×10^6 living bacilli twenty-six days after the immunising dose. The result of the experiment is given below (Tables II and III).

TABLE II.—(Group I.)

Rabbit No. 6.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{2}$ Roux bottle killed bacilli	1820 grm.	*Nil*		
8/9/20		1680 ,,			
11/9/20		1800 ,,	1 in 80		
15/9/20		1850 ,,			
18/9/20		1890 ,,	1 in 80		
22/9/20		1960 ,,			
23/9/20				500×10^6 intravenously	Survived.

Rabbit No. 7.

4/9/20	$\frac{1}{5}$ Roux bottle killed bacilli	1445 grm.	*Nil*		
8/9/20		1640 ,,			
11/9/20		1640 ,,	1 in 40		
15/9/20		1710 ,,			
18/9/20		1730 ,,	1 in 40		
22/9/20		1870 ,,			
23/9/20				500×10^6 intravenously	Survived.

Rabbit No. 9.

4/9/20	$\frac{1}{10}$ Roux bottle killed bacilli	1490 grm.	*Nil*		
8/9/20		1560 ,,			
11/9/20		1530 ,,	1 in 20		
15/9/20		1530 ,,			
18/9/20		1560 ,,	1 in 20		
22/9/20		1730 ,,			
23/9/20				500×10^6 intravenously	Paralysed. Died on 6th day.

Rabbit No. 21 (*Control*).

23/9/20		1900 grm.		1000×10^6 intravenously	Died on 3rd day.

Rabbit No. 22 (*Control*).

23/9/20		1840 grm.		500×10^6 intravenously	Paralysis of hind legs on 3rd day. Recovered.

Post-mortem Findings.

No. 9.—Small intestine showed some œdema but no hæmorrhages. Large intestine appeared normal. Some hæmorrhages in appendix. Lungs, spleen, liver, kidney showed no macroscopic change.

No. 21 (control).—Some small hæmorrhages in right lung. Spleen, liver, kidney and suprarenals appear normal. Small intestine œdematous and injected. Large intestine shows no macroscopic change. Rabbit had coccidiosis.

TABLE III.—(Group II.)

Rabbit No. 10.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{10}$ Roux bottle killed bacilli	1380 grm.	*Nil*		
8/9/20		1500 „			
11/9/20		1510 „	1 in 80		
15/9/20		1500 „			
18/9/20		1520 „	1 in 80		
22/9/20		1680 „			
26/9/20				1000 × 10^6 intravenously	Paralysis of hind legs. Died 3rd day.

Rabbit No. 11.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{5}$ Roux bottle killed bacilli + 8·0 c.c. ox bile	1370 grm.	*Nil*		
8/9/20		1340 „			
11/9/20		1330 „	1 in 20		
15/9/20		1420 „			
18/9/20		1410 „	1 in 20		
22/9/20		1460 „			
26/9/20				1000 × 10^6 intravenously	Survived.

Rabbit No. 12.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
4/9/20	$\frac{1}{5}$ Roux bottle killed bacilli + 8·0 c.c. ox bile	980 grm.	*Nil*		
8/9/20		860 „			
11/9/20		860 „	1 in 40		
15/9/20		850 „			
18/9/20		855 „	1 in 40		
22/9/20		930 „			
26/9/20				1000 × 10^6 intravenously	Survived.

TABLE III.—(Group II)—*continued.*

Rabbit No. 23 (*Control*).

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living *B. dysenteriæ* (Shiga).	Result.
26/9/20.		1780 grm.		1000×10^6 intravenously.	Died 2nd day.

Post-mortem Findings.

No. 10.—Lungs, kidney and suprarenals showed no change. Liver markedly congested. Small intestine, injection and œdema of mucous lining. Rabbit infected with coccidiosis.

No. 23.—Lungs show some small hæmorrhages. Congestion of liver, kidney and spleen. Large intestine showed no change. Small intestine injected and œdematous. No hæmorrhages to be seen macroscopically. Rabbit infected with coccidiosis.

It would appear, then, from these experiments that a certain degree of immunity to *B. dysenteriæ* (Shiga) can be produced in rabbits by the oral administration of killed vaccines, but the experiment as a whole is not completely satisfactory, owing to the failure (in Group 1) of 500×10^6 to kill the control and to the complicating coccidiosis infections. The ingestion of the killed bacilli resulted in the appearance of agglutinins in the serum in every case, but the titre was never a high one. Furthermore, a perusal of these figures shows that the degree of immunity attained does not run parallel with the titre of agglutinins. It will be seen also from the body weights recorded in these two tables, that the ingestion of enormous quantities of killed dysentery bacilli is practically without effect on the general health of the animal. Before proceeding to record the second series of experiments, some observations on the effects of ingestion of living dysentery bacilli and of repeated doses of the killed organism are appended.

Effect of Ingestion of Living B. dysenteriæ (Shiga) on the Rabbits.

In order to ascertain whether or not rabbits could be infected by the introduction *per os* of living bacilli, half a 24-hour Roux bottle culture of living dysentery bacilli was given to a rabbit by the mouth in one dose. The body weight, which on the day of ingestion, 11/9/20, was 1560 grm., fell to 1400 grm. on the 19th of the same month, and by 11/10/20 had still further declined to 1260 grm. Otherwise the animal appeared perfectly well. The examination of its serum for the presence of antibody (agglutinins) gave negative results throughout. Even the administration of large quantities of living dysentery bacilli to rabbits by the mouth is without effect. Similar investigations carried out by Shiga (1898) and Flexner and Sweet (1906) gave negative results also.

Result of a Series of Oral Immunisation Doses.

To determine the degree of immunity produced by a series of doses *per os*, three rabbits were taken (Nos. 2, 3 and 4) and given three doses of $\frac{1}{5}$ of a Roux bottle culture at intervals of ten days. Unfortunately rabbit No 1 died four days after the second dose from pneumonia, associated with *B. pyocyaneus*,

and rabbit No. 4 became infected with an organism of the hæmorrhagic septicæmia group, and had to be discarded. The remaining animal, No. 3 (1780 grm.) was inoculated intravenously with 2000×10^6 living *B. dysenteriæ* (Shiga) on the twenty-fifth day after the last ingestion of vaccine, and at the same time a control rabbit (1700 grm.) received the same dose. The control animal died the third day after the test dose, and rabbit No. 3 became paralysed on the third day and died on the fourth. The post-mortem findings in the control rabbit were much more severe than in the immunised one. Whereas No. 3 merely showed some œdema of the small intestine with one small hæmorrhage in the appendix, the control showed injection and a more marked œdema of the intestinal mucous membrane, with numerous hæmorrhages in the ascending colon, in which was also some diphtheritic ulceration. The lungs showed some small hæmorrhages, and the liver, kidneys, suprarenals and spleen were acutely congested, the latter organ being much enlarged (three times the normal size). *B. dysenteriæ* (Shiga) was isolated from the gall-bladder and spleen, but not from the contents of the large intestine. Although the post-mortem findings point to much more extensive damage in the control rabbit, it must be admitted that none of the other control animals employed in this investigation showed such marked changes. This experiment must be considered, therefore, as inconclusive.

SECOND SERIES OF EXPERIMENTS.

Comparison between the Immunity produced by Ingestion and by Subcutaneous Inoculation.

Since the discovery of *B. dysenteriæ* by Shiga, a practical method of prophylactic inoculation against dysentery has been the subject of many investigations. The high degree of toxicity of this organism has rendered the solution of this problem difficult, and many efforts have been made to reduce the toxicity of the vaccine without interfering with its power to confer immunity. Shiga conceived the idea of inoculating a mixture of killed bacilli and anti-serum. The introduction of sensitised vaccines on the principle of Besredka carried the idea a stage further, and experiments in this connection by Dopter (1909) and Broughton-Alcock (1914) may be here cited. The latter reported good results obtained with a vaccine, the toxicity of which had been reduced by treating the bacterial bodies with heated normal serum. Gibson (1917), Ruffer and Willmore (1918) amongst others have added to our knowledge in this field, and more recently the efficacy of lipo-vaccines has been investigated by Whitmore and Fennel (1918) and Olitsky (1918). In most of this work the titre of antibody has been accepted as a measure of the degree of immunity obtained, but evidence is accumulating that the two do not run a parallel course. From a comparative study of the degree of active immunity produced by different types of dysentery vaccines (heat killed, carbolised, treated with normal or with immune serum), J. D. Thomson (1916) concluded that the carbolised vaccine, whilst giving rise to the least reaction, was productive of the highest degree of immunity. It was decided, therefore, to compare the protection afforded by oral immunisation with that resulting from the subcutaneous inoculation of carbolised vaccines.

Preparation of carbolised vaccine.—This was prepared in the usual manner by suspending the 24-hour growth on agar of *B. dysenteriæ* (Shiga) in 0·5 per cent. carbolic acid in saline. The suspension was counted and the doses required made up to 1·0 c.c. with saline as required.

Administration of vaccine per os.—In these experiments the vaccine was delivered into the stomach by means of a small rubber cannula to which was affixed a small filter funnel. In this experiment 24 rabbits of suitable size were taken and divided into 2 series of 12 each. Series 1, which received the vaccine *per os*, was further subdivided into two groups, A and B, which were treated as follows:

SERIES I.

Group A : Rabbits Nos. 27, 28, 29, 30, 31 and 32.

Each rabbit in this group received three doses by the oral route of a suspension of *B. dysenteriæ* (Shiga), killed by heating to 60° C. The dose in each case was $\frac{1}{5}$ of a Roux bottle culture and the interval between the dose ten days.

Group B : Rabbits Nos. 33, 34, 35, 36, 37 and 38.

These rabbits were immunised by the oral route, receiving similar doses to those given to Group A, with, however, the addition of 8·0 c.c. of sterile ox bile with each dose of vaccine. One of these animals—No. 35—died three days after the first dose from pneumonia.

SERIES II.

This series consisted of 12 rabbits which were immunised by subcutaneous inoculation of a carbolised vaccine of dysentery bacilli (Shiga). Three doses were given, one of 50 million and two of 100 million, the interval between the doses being ten days. Rabbits Nos. 40 and 52 died five days after the first inoculation, post-mortem examination revealing some congestion of the right lung, with œdema and injection of the small intestine.

The surviving animals of these two series were tested for their immunity to living *B. dysenteriæ* (Shiga) three weeks after the last immunising dose. The test doses employed were 1000 millions and 2000 millions, and were given intravenously. The results of this experiment are tabulated below:

TABLE IV.

No. of rabbit.	Immunisation.	Test dose.	Result.
27	3 doses ($\frac{1}{5}$ Roux bottle) per os	1000 million	Died on the 5th day.
28	,,	,,	Died on the 5th day.
29	,,	,,	Survived.
33	,, + ox bile	,,	Died on the 5th day.
34	,, ,,	,,	Died on the 5th day.
30	3 doses ($\frac{1}{5}$ Roux bottle) per os	2000 million	Survived.
31	,,	,,	Died on the 2nd day.
32	,,	,,	Died on the 5th day.

TABLE IV—*continued.*

No. of rabbit.	Immunisation.	Test dose.	Result.
36	3 doses ($\frac{1}{5}$ Roux bottle) + ox bile	2000 million	Died on the 2nd day.
37	,, ,,	,,	Survived.
38	,, ,,	,,	Paralysed—recovered.
39	Subcutaneous inoculation of carbolised vaccine, 3 doses (50, 100 and 100 million)	1000 million	Survived.
41	Ditto	,,	Survived.
42	,,	,,	Survived.
43	,,	,,	Survived.
44	,,	,,	Survived.
47	,,	2000 million	Survived.
48	,,	,,	Survived.
49	,,	,,	Survived.
50	,,	,,	Survived.
51	,,	,,	Died on the 5th day.
	Controls (Body weight.)		
54	2000 grm.	500 million	Survived.
57	1450 ,,	,,	Died on the 3rd day.
55	1860 ,,	1000 million	Died on the 2nd day.
58	1974 ,,	,,	Died on the 2nd day.
56	1700 ,,	2000 million	Died on the 2nd day.
59	2000 ,,	,,	Died on the 3rd day.

The surviving rabbits were observed over a period of three weeks. During the first two weeks there was some loss of weight, but by the end of the third week an increase in weight was recorded.

Post-mortem findings.—In the case of the control rabbits the mucous membrane of the small intestine was œdematous and injected. Nos. 55, 58 and 59 showed some hæmorrhages in the lungs. Those rabbits immunised *per os* which succumbed to the test-dose showed the same changes as the control animals though they were less acute. Rabbit No. 51, the only animal of Series II which died after receiving the test dose, showed rather severe changes. Hæmorrhages were present both in the lungs and large intestine.

DISCUSSION.

From the above experiment it will be seen that while the rabbits immunised *per os* had acquired a certain degree of resistance to the intravenous inoculation of *B. dysenteriæ* (Shiga) (3 survivals out of 11 possibles), a much more solid immunity (9 survivals out of 10 possibles) had resulted from the subcutaneous inoculation of a carbolised vaccine. It is noteworthy also that the ingestion of bile did not seem to increase the efficacy of oral immunisation. Besredka drew attention to this point also, and states that the addition of bile was without result in the immunisation against *B. dysenteriæ*. It might be pointed

out also, that in the first series the oral immunisation was carried out by feeding the rabbits with the killed bacilli, whereas in the second series the vaccine was introduced into the stomach by means of a cannula. In neither case did any solid immunity result.

CONCLUSIONS.

(1) It has been possible to produce a certain small degree of immunity in rabbits by the oral administration of *B. dysenteriæ* (Shiga).

(2) The immunity so obtained is found to be far inferior to that produced by the subcutaneous inoculation of a carbolised vaccine administered in three doses of 50, 100 and 100 millions.

(3) The oral administration of large quantities of killed dysentery bacilli to rabbits is without effect on their general condition as judged by the body weight.

To many members of the Bacteriological Staff of this Institute I am indebted for helpful advice and criticism.

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THE COMPARATIVE INFLUENCE OF PURE AND COMMERCIAL SUGARS AND OF COMBINED AND SEPARATE STERILISATION ON BACTERIAL METABOLISM.*

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For a long time bacteriologists who have been concerned with the differentiation of bacterial groups or subgroups by means of fermentation reactions have attempted to ensure the normality of their results by securing sugars from well-known makers of fine chemicals. In very few cases, indeed, have the sugars been re-purified or their purity tested by the workers examining them.

When one realises the technical difficulties of purification and of analysis this is quite understandable, and the samples of sugars, such as glucose and levulose, sent out by the best firms as chemically pure go to show that even the makers have not found the task of purification easy.

Most workers have recognised that the sugars with which they worked were not chemically pure even when so labelled. The colour and turbidity of the solutions were evidence of this. It can be said generally that the purity of carbohydrates as bought in the market is far behind the standard exacted for a similar class of inorganic chemicals.

The Carbohydrate Laboratory of the Department of Agriculture of the United States has recognised the disability of bacteriologists in this regard, and has sought to remove it by devoting some time to the methods of carbohydrate purification. This has been done with such success that it is now easy to prepare glucose (Hudson and Dale), fructose (Hudson), xylose (Hudson and Harding), galactose (Mason) and other sugars in a very high state of purity by the methods of Hudson and his collaborators.

Certain firms are also taking up the preparation of pure sugars and endeavouring to set standards of purity. Two recent papers, one by Pfanstiehl and Black (1921) and another by Graber (1921), give an indication of what can be done in this respect. With Schmit-Jensen (1920) they point out that none of the chemical tests are to be compared in delicacy with the biological tests. Conversely, therefore, sugars should be in a high state of purity if they are to be used for detecting fine differences in bacterial behaviour.

That small amounts of impurities may affect fermentation reactions is

* A report to the Medical Research Council.

shown by the recent work of Schmit-Jensen, who has experimented with the rarer types of sugar by means of his ingenious micro-fermentation tubes. By the use of an organism such as *B. suipestifer* acting on xyloses he has been able to show that so small a concentration as 0·025 per cent. in bouillon will manifest itself by the production of gas.

With regard to the uncertainty existing in the differential fermentation of bacteria, it is difficult to make out whether the differences obtained by individual workers have been due to impurities in the sugar employed, to variations in the methods of preparing the media or to variations in the organisms themselves. We know that in the preparation of so complex a mixture as a bacteriological medium many factors enter which are difficult to control. One has only to recall the profound difference obtained by using different samples of peptone in the preparation of diphtheria toxin to realise that even now many of these factors are not resolved.

Certain workers have, however, paid some attention to the question of the purity of sugars employed for bacteriological purposes. Elser and Huntoon (1909), in their study of the meningococcus, go into the matter at some length. They used samples of sugar obtained from Kahlbaum and from Merck, without, however, attempting to purify them or to analyse them. They point out the ease with which galactose is altered by treatment with the smallest quantities of alkali, and that, in the case of dextrin, the various samples of this carbohydrate gave quite different results, due, as they believe, to the presence of impurities. It will be noted that dextrin has given anomalous results with the meningococcus in the hands of different observers.

Inulin is another carbohydrate of great utility when pure, but it is so often contaminated with fructose that a pure sample is difficult to obtain. The time of harvesting the artichokes is of great importance in securing the pure polysaccharide.

Differences in the behaviour of the gonococcus with galactose have been observed by various workers, and Elser and Huntoon showed that the fermentation observed by Dunn and Gordon did not take place if the sugar was sterilised separately from the bouillon, and the mixture effected aseptically after sterilisation of both.

With *B. diphtheriæ* similar differences have been encountered: thus Simmons (1920) found organisms with all other characteristics similar from human and feline cases of diphtheria which behaved differently to saccharose.

With regard to the influence of sterilisation on media containing glucose, there is equal reason to pre-suppose that one might find differences in the behaviour of media prepared in the ordinary routine way and those to which the sterile sugar solution has been added after sterilisation of the bouillon. I have observed in previous experiments that there is a loss in amino-acid content when sugar and tryptic broth are sterilised together, and have explained it as partly due to the Maillard (1913) type of condensation of amino acid and glucose. This loss is well seen in the present experiments. The change in hydrogen ion concentration was detected by the older bacteriologists in titrating media before and after sterilisation, and has been recorded many times since. That the effect of combined sterilisation may have important practical bearings is shown by the results of Elser and Huntoon on the gonococcus, and especially

the meningococcus. With the latter organism no fermentation took place with either galactose or fructose when the sugar was sterilised separately in solution in distilled water. Lobry de Bruyn and Alberda van Ekenstein (1895) have shown that small quantities of alkali and heat will change a part of fructose to glucose; and, indeed, Kendall, Day, Walker and Ryan (1919) have recently asserted that it is useless to perform tests with fructose when the results with glucose are known, owing to the stereo-chemical lability of these sugars when the solvent is alkaline (see Wohl, 1893, 1897).

In view of the above it seemed worth while to ascertain whether, with some of the more common organisms, a quantitative difference could be made out when pure and commercial sugars were employed. As the sterilisation of sugars with the medium had been shown to yield definite qualitative differences with organisms such as the meningococcus, an investigation of this factor was undertaken at the same time.

In the course of work which had been done in my laboratory on the fermentation of organisms, it has been found that a very good degree of quantitative uniformity could be made out when the same organism was allowed to ferment under identical conditions. The quantitative method ought therefore to show up any difference due to the purity or treatment of the carbohydrate employed which would not be apparent with ordinary fermentation tests.

I was fortunate, through the kindness of Dr. C. F. Walton, Jr., of the Carbohydrate Laboratory in Washington, in being able to obtain from him a sample of chemically pure glucose prepared by the method of Hudson. This was used in part of the work. In addition I recrystallised a good sample of "chemically pure" glucose from glacial acetic acid by this method. The recrystallisation was repeated. The product did not differ from that supplied me by Dr. Walton. It was snow-white, and gave an absolutely water-white solution when dissolved in water. It had been heated to 80° C., and subsequently kept *in vacuo* over sulphuric acid and potassium hydroxide for a long time.

The lactose was prepared from the sample which was used as "commercial" lactose. It was recrystallised three times from boiling distilled water after decolorisation with animal charcoal. This sugar also gave an absolutely water-white solution.

The commercial samples of glucose and lactose were obtained from a well-known chemical supply firm, and came labelled "chemically pure." The glucose had a distinct yellow tinge. The solution of the sugar was light brown in colour. The lactose had much the same external qualities as the glucose. Both samples were of the quality usually employed in bacteriological laboratories.

In the preparation of the sugars for addition to the medium the following method was employed: A 20 per cent. solution of glucose was made up in distilled water, and made faintly acid to Congo red with hydrochloric acid. This was tubed off in lots of 20 c.c. and sterilised at 120° C. for 30 minutes.

The lactose was also dissolved in the same concentration in distilled water and sterilised without the addition of acid. Both solutions were colourless after treatment with heat. The commercial sugars were treated in the same way. They gave light to darkish brown solutions. The fermentations were

carried out in long-necked 600 c.c. kjeldahl flasks (Harris, 1919). Where sugars were added after sterilisation of the medium, this was done aseptically by adding the contents of the sugar tube to the medium. The flasks were allowed to stand at 37° C. for 24 hours to test for sterility. After fermentation any evaporation was allowed for by diluting the fermented media to a definite volume. With the exception of *B. murisepticus* all fermentations were carried out anaërobically in order to follow easily the gas evolution.

The organisms employed were *B. coli*, *B. welchii*, *B. murisepticus* and *B. tetani*.*

These organisms were chosen because in *B. coli* and *B. welchii* we have bacteria which are not sensitive to the *milieu* in which they develop. *B. tetani* and *B. murisepticus* are usually assumed to be of somewhat fastidious growth habit.

The method of testing was to set up a series of flasks so that under identical conditions and with the same quantity of inoculum there were the following media:

(1) Basic medium with pure sugar added aseptically.
(2) Basic medium and pure sugar sterilised together.
(3) Basic medium with commercial sugar added aseptically.

The three flasks were kept at a constant temperature in one thermostat. In another at the same temperature duplicates of (1) and (2) were fermented.

The basic medium used was the tryptic broth of Cole and Onslow, which was diluted 1 : 3, giving a nitrogen content for the finished medium of about 0·3 per cent.

Table I (experiments A–E) gives the results of two sets of tests with pure glucose and *B. coli* where the sugar had been added before sterilisation (A and C) and after sterilisation (B and D) with a control when ordinary glucose had been used (E). For the inoculum a culture of *B. coli* was used which had been passed twice through peptone at 24-hour intervals and once at a 10-hour interval.

FERMENTATION OF *B. COLI* IN GLUCOSE PEPTONE.

The effect of sterilisation on the medium.—As a result of combined sterilisation one finds that the ammonia and volatile acids are slightly higher, but the most pronounced effect is that on the amino-acid concentration. There is a very marked disappearance of amino-acid groups as the result of heating the nitrogen containing molecules with the sugar. The extent to which this occurs depends apparently on the composition of the medium, the extent of heating, and other factors which have not been studied, for in the second glucose medium used for *B. welchii* the process has not gone on so far. This is probably due to the amount and character of the preliminary tryptic hydrolysis. A discussion of this would lead far, but the recent experiments of Onslow (1921) seem to show that tryptic digestion is not easily controlled, for some workers obtain yields of free

* For *B. murisepticus* and *B. tetani* I am indebted to Dr. J. C. G. Ledingham, Director of the National Collection of Type Cultures, Lister Institute. *B. coli* was a human type isolated by Dr. Wyeth in my laboratory and used by him in his studies of this organism. *B. welchii* was one of the strains used by me in former work.

tryptophane on simple tryptic digestion which in other circumstances are only given by a second digestion. Onslow's experience has been confirmed in this laboratory.

The volatile acids are higher, as are the fixed acids. To this may be ascribed the increase in hydrogen ion concentration. In this experiment the volatile acids have roughly been doubled as the result of sterilisation.

The condensation of glucose is shown by a loss of 0·07 per cent. on an initial concentration of 1·87 per cent.

The effect of sterilisation on fermentation.—While with the added sugar, both pure and commercial, there is a marked consumption of amino nitrogen, amounting in the former case to 40 mgrm., the amount of this component is larger at the end of fermentation when sugar and medium have been sterilised together. It would appear that the organism has attacked the condensation products of sugar and amino acids, and liberated part of the latter. The two experiments are quite concordant. Volatile acid formation is always higher where sugar has been added separately.

The composition of the evolved gases varies within the fairly narrow limits of 49·8 and 53·8 per cent. for carbon dioxide. It does not appear that sterilisation of the sugar with the tryptic broth has influenced this phase of the reaction. The total evolution of gas also does not seem to have been influenced either by sterilisation or by the quality of the sugar. The total variation is 43 c.c., the highest amount being evolved by the impure glucose.

No real difference in final reaction is to be made out in the various experiments, the resulting pH being 4·6. The consumption of sugar is slightly less in the experiment where glucose and medium have been sterilised together.

FERMENTATION OF B. COLI WITH LACTOSE PEPTONE.

The influence of sterilisation on the medium.—As shown in Table II, the effect on the lactose medium, as might be expected, is not so pronounced as with glucose, but, in general, heating has produced changes in the same direction. The ammonia is slightly lower in the combined sterilisation, but the change is not well marked in any. There is a small disappearance of amino acid in this medium, but this is confused by the result obtained with impure glucose where the amount of amino nitrogen is lowest. It will be noted that the results obtained in the second experiment with *B. welchii* in this medium are slightly in the other direction. It is therefore safe to assume that any effect on the amino nitrogen is negligible.

The volatile acids are undoubtedly higher, agreeing well with the control media in the *B. welchii* experiment. The fixed acids are practically unchanged by the heating. There has been a destruction of lactose as the result of heating, amounting to about 0·2 per cent. There is not the same increase in hydrogen ion concentration observed with the glucose medium, the increase amounting to $pH = 0·3$. In the *B. welchii* experiment the difference was $pH = 0·4$.

The effect of sterilisation on fermentation.—The amount of gas evolved does not seem to have been influenced by sterilisation or by the impurity of the

TABLE I.—B. coli *in* 2 *per cent. Glucose-peptone.*

	Pure glucose and peptone sterilised together.			Pure glucose and peptone sterilised separately.			Commercial glucose and peptone separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent. . . .	0·325	0·321	0·323	0·327	0·323	0·332	0·324	0·336
Ammonia nitrogen: mgrm. per 100 c.c.	19·05	29·0	30·0	15·9	32·1	30·8	18·2	31·8
Amino acid nitrogen: mgrm. per 100 c.c.	80·0	96·4	96·2	120·5	80·0	80·6	118·0	90·7
Volatile acids: c.c. N/10 acid per 100 c.c.	7·4	20·7	21·6	3·8	25·2	22·2	3·61	23·5
Fixed acids: c.c. N/10 acid per 100 c.c.			26·3					
Carbon dioxide per cent. . . .		50·9	50·8		53·8	50·6		49·8
Hydrogen per cent.		49·1	49·2		46·2	49·4		50·2
Total gas: c.c. in 47 hours . . .		241	232		221	253		264
pH 20° C.	6·60	4·64	4·64	7·15	4·70	4·65	7·20	4·69
Glucose	1·89	1·22	1·22	1·87	1·25	1·20	1·86	1·19

TABLE II.—B. coli *in* 2 *per cent. Lactose-peptone.*

	Pure lactose and peptone sterilised together.			Pure lactose and peptone separately.			Commercial lactose and peptone separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent. . . .	0·336	0·326	0·326	0·320	0·333	0·326	0·314	0·342
Ammonia nitrogen: mgrm. per 100 c.c.	17·9	30·2	30·9	19·0	21·2	31·1	18·6	26·7
Amino acid nitrogen: mgrm. per 100 c.c.	120	109	105	125	116	119	111	118
Volatile acids: c.c. N/10 acid per 100 c.c.	5·5	26·7	23·4	3·4	25·5	24·2	3·2	25·7
Fixed acids: c.c. N/10 acid per 100 c.c.	13·1	41·7	42·0	14·5	41·7	46·7	9·0	41·2
Carbon dioxide per cent. . . .		46·1	46·1		52·1	45·7		52·5
Hydrogen per cent.		53·9	53·9		47·9	54·3		47·5
Total gas: c.c. in 48 hours . . .		286	282		304	294		280
pH 20° C.	6·92	5·01	4·96	7·22	5·01	4·93	7·25	5·00
Lactose	1·54	1·11	1·11	1·72	1·13	1·15	1·69	1·21

glucose. It is of the same order as that produced from glucose. The final concentration of sugar is nearly the same in all experiments with the exception of the commercial glucose, where it is slightly higher. Owing to the initial concentration of lactose in the combined sterilised medium being low, the consumption of sugar in this medium is less than in the one where sugar was added before sterilisation.

FERMENTATION OF B. WELCHII WITH GLUCOSE PEPTONE.

The inoculum was prepared by transferring *B. welchii* through two meat tubes to milk for 24 hours, and from this medium to glucose peptone for 14½ hours; 0·7 c.c. was used for inoculation. The growth was somewhat slimy in character.

The effect of fermentation on the production of ammonia in the pure glucose media is entirely negligible (Table III), while there is an increase in the amount of amino nitrogen, due to the action of the bacteria. The amino-acid formation is the same in amount in both combined and separate media. The result with ordinary glucose is not decisive, both initial and final values being the same. The amount of volatile acids is not sufficiently affected to show any difference due to the method of sterilisation, and the same may be said of the fixed acids. In one respect, however, combined sterilisation has affected the fermentation, and that is in the gas production. Here it is considerably lower, then, when glucose is added separately to the medium. *B. welchii* is considerably more sensitive in this respect than *B. coli*.

The final amounts of sugar remaining over after fermentation are the same. With the commercial sugar experiment, as the initial concentration in the combined sterilisation is lower, less sugar was consumed in these experiments.

The final hydrogen ion concentrations are all alike.

FERMENTATION OF B. WELCHII IN LACTOSE PEPTONE.

The results (Table IV) in this experiment do not call for any special comment. There are no striking differences to be made out. Both nitrogen and carbon metabolism do not appear to have been affected by changes in the medium due either to the purity of the carbohydrate or to the method of sterilisation. There appears to have been less sugar consumption and a lower hydrogen ion concentration in the medium where the sugar was sterilised separately from the bouillon.

FERMENTATION OF B. MURISEPTICUS IN GLUCOSE PEPTONE.

In this experiment (Table V), which was allowed to continue for fifteen days, only the effect of purity of the sugar was examined. Here a certain difference was encountered. The ammonia production was considerably higher when commercial glucose was employed, while the volatile acid production was slightly lower. I was unable to determine the fixed acid production. The resulting final hydrogen ion concentration of the experiments was absolutely in agreement.

TABLE III.—B. welchii *in* 2 *per cent. Glucose-peptone.*

	Pure glucose and peptone sterilised together.			Pure glucose and peptone sterilised separately.			Commercial glucose and peptone sterilised separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent.	0·325	0·325	0·321	0·319	0·332	0·334	0·320	0·328
Ammonia nitrogen: mgrm. per 100 c.c.	23·2	24·2	21·7	27·1	26·7	30·4	19·0	29·2
Amino acid nitrogen: mgrm. per 100 c.c.	99·4	110·5	106·6	109·1	124·6	121·0	121·5	122·0
Volatile acids: c.c. N/10 acid per 100 c.c.	5·54	34·1	29·8	2·26	30·3	31·0	2·8	?
Fixed acids: c.c. N/10 acid per 100 c.c.	9·8	28·2	41·5	8·4	34·9	37·5	9·0	37·5
Carbon dioxide per cent.		45·4	44·4		45·2	45·4		47·2
Hydrogen per cent.		54·6	55·6		54·8	54·6		52·8
Total gas: c.c. in 24 hours		267	300		410	400		415
pH 20° C.	6·17	4·43	4·46	6·90	4·43	4·45	6·95	4·48
Glucose	1·62	0·98	0·99	1·70	0·98	1·00	1·80	0·69

TABLE IV.—B. welchii *in* 2 *per cent. Lactose-peptone.*

	Pure lactose and peptone sterilised together.			Pure lactose and peptone sterilised separately.			Commercial lactose and peptone sterilised separately.	
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.	Initial.	Final.
Total nitrogen per cent.	0·206	0·208	0·210	0·201	0·208	0·212	0·203	0·216
Ammonia nitrogen: mgrm. per 100 c.c.	15·3	8·9	19·5	14·8	31·3	24·3	15·4	36·6
Amino acid nitrogen: mgrm. per 100 c.c.	98·0	111·0	109·0	93·8	98·6	103·0	98·5	103.0
Volatile acids: c.c. N/10 acid per 100 c.c.	4·0	29·6	28·6	2·5	30·8	30·2	2·5	36·2
Fixed acids: c.c. N/10 acid per 100 c.c.	4·6	30·8	30·7	3·8	28·8	32·8	7·65	34·5
Carbon dioxide per cent.		43·7	47·2		44·9	46·5		47·2
Hydrogen per cent.		56·3	52·8		55·1	53·5		52·8
Total gas: c.c. in 48 hours		405			425	421		420
pH 20° C.	6·80	4·72	4·69	7·22	4·98	5·00	7·15	5·01
Lactose	1·60	1·09	1·11	1·62	1·26	1·24	1·59	1·25

TABLE V.—B. murisepticus *in* 1 *per cent. Glucose-peptone.*

	Pure glucose and peptone.		Commercial glucose and peptone.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·220	0·220	0·220	0·220
Ammonia nitrogen: mgrm. per 100 c.c.	13·6	53·3	13·6	78·5
Amino acid nitrogen: mgrm. per 100 c.c.	100·0	108·5	100·0	107·7
Volatile acids: c.c. N/10 acid per 100 c.c.		18·2		15·5
pH		4·60		4·60

FERMENTATION OF B. TETANI IN GLUCOSE PEPTONE.

Two experiments were made with *B. tetani*, one lasting 143 hours and the other lasting 323 hours (Table VI). The organism was sown on cooked meat and an inoculum of 0·6 c.c. used.

TABLE VI.—B. tetani *in* 1 *per cent. Glucose-peptone.*

	Pure glucose and peptone.			Commercial glucose and peptone.		
	Initial.	Final I.	Final II.	Initial.	Final I.	Final II.
Total nitrogen per cent.	0·242	0·238	0·223	0·247	0·239	0·212
Ammonia nitrogen: mgrm. per 100 c.c.	15·1	65·1	59·3	15·4	65·0	64·0
Amino acid nitrogen: mgrm. per 100 c.c.	110·0	49·3	71·1	113·0	49·4	74·2
Volatile acids: c.c. N/10 acid per 100 c.c.		32·0	28·5		31·7	31·0
Total gas evolved: c.c.		44·4	64·2		39·7	63·2
Carbon dioxide per cent.			61·3			64·1
Hydrogen per cent.			38·7			35·9
pH						6·9

The fermentation, as shown by gas evolution, was very slow, only about 3 to 5 c.c. of gas being produced in the first twenty-four hours. From thereon the course of gas production rose until about the fiftieth hour, when evolution slowed up. The gas production in the second experiment was always somewhat in advance of the first. They were not conducted with the same inoculum. The noteworthy feature of the fermentation of *B. tetani* is the high ammonia formation and amino-acid consumption. This is typical of strongly proteolytic organisms, such as *B. sporogenes* and *B. histolyticus* (Wolf and Harris, 1918). There is no difference to be observed between the fermentations conducted with pure and with commercial sugars.

SUMMARY.

Four organisms, *B. coli*, *B. welchii*, *B. murisepticus* and *B. tetani*, have been examined to ascertain whether the purity of a sugar or the combined

sterilisation of the sugar with the bouillon had an effect on the products of bacterial metabolism. The sugars used were glucose and lactose. While minor differences were encountered, these were not sufficient to indicate that combined sterilisation or commercial glucose or lactose would influence a fermentation reaction sufficiently to lead to error. At the same time it must be remarked that with organisms more sensitive than those employed in the present study it is quite possible that such errors could take place.

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SEPTICÆMIA: THE SELECTIVE DEPOSITION OF THE COLON TYPHOID GROUP OF BACTERIA IN FIXATION ABSCESSES.

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THE constancy of the septicæmic stage early in an attack of typhoid fever, before extensive localising lesions are to be found, makes it probable that this organism can enter and presumably leave the blood-stream without the help of such gross mechanical lesions as would occur where thrombosis of vessels and suppurative disintegration of tissues has taken place. The great frequency of *B. coli* infections of the urinary tract, many of which are most probably hæmatogenous in origin, and which often set in without any marked general symptoms, further goes to show that the coliform bacilus also can enter and leave the blood-stream with comparative ease.

In the experiments detailed below it is shown that the coli-typhoid group of bacteria can in fact pass out of the blood-stream into certain materials introduced into the subcutaneous tissues, and continue their growth there, while other bacteria present in the blood-stream fail to do so.

Inasmuch as this group of organisms, whether in health or disease, is to be found growing in the mucus-secreting intestinal canal the fixation areas were first made with a mucoid substance—gum tragacanth—and were designed to find whether the coli-typhoid bacteria as distinguished from other organisms were specifically thrown out from the blood-stream into this substance. With few exceptions this seemed to be the case.

Preparation of the gum.—An emulsion of about 5 per cent. gum tragacanth in water will just pass through a fair-sized exploration needle, and this strength was used in all the experiments. The emulsion was made up in the usual way by moistening the powder with alcohol, and then rapidly mixing with the appropriate amount of water and shaking well. The emulsion is spoiled by autoclaving; and, in fact, to be ideal, should not be heated at all, but, if necessary, it can be sterilised by steaming for a few minutes on three consecutive days. Almost all the samples I have worked with have been infected with sporing bacilli.

Experiment 1.—The Fate of the Sterile Gum in the Subcutaneous Tissues.

When first injected the gum forms under the skin a rounded elastic lump, which in the course of a few days swells up a little and becomes more diffuse,

then diminishes in size and becomes firmer, but does not entirely disappear. It ultimately becomes replaced with a mass of fibrous tissue, which may contain a small area of degenerate pus. As shown by puncture and examination of films, invasion by phagocytes begins within an hour or so after the injection, and becomes more intense as time goes on, although as a rule the mucoid character is retained for several weeks. If the animal is killed the day following injection leashes of dilated vessels are seen all over and about the gum, associated with a good deal of œdema.

Experiment 2.—Injection of Gum Infected with Living Bacteria.

The results to be obtained in this experiment vary considerably according to what organism is being used. Infection with living *B. coli* shows that this bacillus grows freely in the mass despite the leucocytic infiltration. The abscess grows larger in the first few days and softens, although it rarely breaks down sufficiently to involve the skin. After the first few days of active infiltration the abscess, as a rule, shrinks up during the succeeding two or three weeks, and then becomes partly or wholly organised. The definitely mucoid character of the contents is usually still marked up to the end of the first week.

Infection of the gum with living *S. aureus* gives a more active picture. The infiltration with phagocytes from the first is much more fierce; there is extensive œdema in the vicinity, and masses of lymph are deposited throughout the abscess, which is liable in a few days to break through the skin, discharge freely, and heal up completely. The staphylococcus, like the coliform bacillus, appears to multiply in the gum in the first day or two after injection. In both staphylococcal and coliform abscesses phagocytosis may be noted to a variable extent during the second or third day, but it is not usually a notable feature. It is noteworthy that while the coliform bacilli seem to remain unchanged as regards their staining characters, the staphylococci within a day or two often lose their capacity to retain Gram's stain, or stain with only a small central dot.

In addition to these two principal types of organisms, the various other bacteria referred to in the later experiments were also inoculated directly into gum masses and injected, and with the exception of the gonococcus they were recovered living from the abscesses on the following day. In most cases they appeared to have increased in numbers.

Experiment 3.—The Migration of B. coli *from the Blood-stream into a Subcutaneous Mass of Gum.*

A rabbit was injected subcutaneously in the flank with 5 c.c. of the sterile gum. At the same time about 2500 million living *B. coli* suspended in 1 c.c. saline were injected into the ear vein. Three hours after injection the gum mass was punctured with a sterile needle on a syringe and some of the contents withdrawn. The mucoid matter was slightly turbid, and films showed a few leucocytes and occasional Gram-negative bacilli. Several colonies of *B. coli* grew from a loopful of it in pure culture. Puncture after 6 hours showed the same conditions in a more marked degree. Puncture 24 hours

after injection showed pus-cells and bacilli in very large numbers, and *B. coli* grew in a massive and pure culture. Occasional punctures showed the presence of living *B. coli* in the abscess up to the end of the second week, by which time the mucoid character had entirely disappeared, and the abscess had a dense fibrous wall with a purulent centre.

Living *B. coli* were obtained from the blood-stream up to the third day after injection.

The experiment shows that *B. coli* may leave the blood-stream and start within three hours to set up a local infection in a mass of sterile gum lying in the subcutaneous tissues. It further shows that *B. coli* may remain alive in the blood-stream for 72 hours, and in a localised abscess of the nature described for at least 2 weeks. (In a previous paper I (1920) have shown that Shiga's dysentery bacillus may be recovered alive from a similar abscess in the guinea-pig after 2 months.)

Organisms contained in a localised abscess of this type are obviously protected from the destructive agencies of the host, and this in spite of the presence of numerous phagocytes.

Experiment 4.—Repetition of Experiment 3, but using S. aureus *in place of* B. coli.

5 c.c. gum were injected subcutaneously in the flank, and 5000 million *S. aureus* were injected simultaneously into the blood-stream. The gum was punctured the next day and again the following day, and examined by film and culture. Large numbers of pus-cells were seen but no cocci, and both times cultures remained sterile. A similar puncture on the fifth day was also sterile. The experiment was repeated on another rabbit, with identical results.

We learn from this experiment that the *S. aureus* does not readily pass out of the vascular system to infect a mass of gum injected into the subcutaneous tissues, and in this respect it is in marked contrast to the coliform bacillus which was found in the gum within three hours after the injection. It seems that some special mechanism is in operation enabling the former to pass through the vessel walls.

Experiment 5.—Repetition of Experiments 3 and 4, using both Types of Bacteria together.

A rabbit was injected with gum as before, and a saline emulsion containing living cultures of *B. coli* and *S. aureus;* 2500 million of each were at the same time given intravenously. Puncture of the gum after 3, 6 and 24 hours showed only bacilli in the abscess, and with the exception of one colony of *S. aureus*, obtained at the second puncture, pure growths of *B. coli* were obtained each time.

As it has been shown in Experiment 2 that either of these types of bacteria is able to grow in the gum when injected into it direct, it follows from Experiments 3, 4 and 5 that a definite selective capacity exists, which enables bacteria of the colon type to leave the blood-stream and infect the gum much more readily than those of the staphylococcal type.

Experiment 6.—Repetition of the previous Experiments, using various other Bacteria.

(*a*) Experiment 5 was repeated, using *B. paratyphosus B* instead of *B. coli*. This organism was recovered from the gum as in the case of *B. coli*, but, as before, the cocci were not found in it.

(*b*) A mixed emulsion of *B. typhosus*, *S. aureus* and Hofmann's bacillus was tested by intravenous inoculation in the same way, and only the typhoid bacillus was obtained from the gum.

(*c*) Other organisms similarly tested as regards transference from the blood-stream to the gum were *M. catarrhalis* and streptococcus, a mixed culture freshly isolated from the sputum. Neither of these organisms was grown in culture from the gum, although a few streptococci were seen in films made 24 and 48 hours after inoculation. Both were grown in blood cultures made 24 hours after injection into the blood-stream.

(*d*) A mixed emulsion of the influenza bacillus and streptococcus, freshly isolated from sputum, was tried, but neither organism was recovered from the gum. On the day following the injection only the streptococcus was grown in blood-culture (3 c.c. of blood).

These experiments, then, show that while the coli-typhoid organisms almost always appear in the fixation gum abscess, the various other bacteria, examined in the same way, almost invariably fail to be transferred from the blood-stream to the fixation area.

The recovery of the coli-typhoid bacilli did not, however, occur on every occasion without exception: thus out of 9 times that Experiment 5 was done *B. coli* was recovered in 5 of the experiments in pure culture from several punctures. In 2 other experiments staphylococci in small numbers were grown from the gum, in addition to *B. coli*. Once the gum remained sterile, and once *S. aureus* only grew from it.

Of 3 experiments with *B. typhosus* in company with *S. aureus*, once the cultures remained sterile and twice typhoid bacilli grew in pure culture from repeated punctures. The two experiments with *B. paratyphosus B* along with *S. aureus* both resulted in pure cultures of paratyphoid bacilli in the fixation abscesses.

Experiment 7.—Substances other than Gum used for the Fixation Areas.

Mucin.—Commercial mucin (scales) dissolved in dilute caustic soda and neutralised with HCl was used as a subcutaneous inoculum. As in the gum experiments, *B. coli* and *B. typhosus* were recovered from the mucin, whilst the staphylococci failed to appear in it. Only two experiments were carried out, as the technical difficulties in preparing the mucin sterile were considerable.

Starch.—A 5 per cent. emulsion of boiled starch was used in 3 experiments as a fixation area, with an intravenous inoculum of mixed *B. coli* and *S. aureus*. After 24 hours the starch showed a considerable number of phagocytes, but no organisms, and cultures remained sterile. The result after 48 hours was the same. In the third repetition of the experiment a few colonies of *B. coli* were grown from a puncture 48 hours after inoculation.

The presence of numerous phagocytes in the starch without any infecting organisms is an interesting comment on a possible suggestion that bacteria might be carried into the gum by these migrating cells.

Agar.—Two experiments were carried out using sterile 2 per cent. agar as the subcutaneous inoculum, injecting it when cold and solid. *B. coli* and staphylococci were also injected intravenously. After 24 hours a fair number of leucocytes had migrated into the agar, but no bacteria were seen and cultures remained sterile. In a second experiment a great number of bacilli were seen in films after 24 hours, and cultures grew an abundance of *B. coli* in pure culture.

McConkey's bile-salt agar.—The two agar experiments were repeated with this medium as a subcutaneous inoculum. Leucocytes migrated into it freely, and in both experiments an abundance of *B. coli* grew from the 24 hours' puncture. In the first experiment the coli were in pure culture, but in the second a few colonies of *S. aureus* also grew.

As control a further rabbit was injected with masses of sterile agar and McConkey's agar; these masses remained sterile but were freely invaded by phagocytes.

Muscle.—The pale hind limb muscles of a freshly-killed rabbit were finely smashed up in a mortar and injected subcutaneously into a rabbit, a mixed suspension of *B. coli* and *S. aureus* being given intravenously at the same time. The rabbit was found moribund 24 hours later and was killed. There was much inflammation in the region of the inoculum with an extensive œdema, which gave the whole district a watery mucoid appearance. The mashed muscle seemed very little changed and films showed only a few leucocytes invading it; large numbers of Gram-negative bacilli were present, but no cocci. *B. coli* grew abundantly in pure culture from the watery exudate. The experiment was not successfully repeated on account of the great difficulty in pushing the mashed muscle through a syringe and needle. This experiment again shows that *B. coli* leaves the blood-vessels more readily than does the staphylococcus, and as in the case of the starch experiment, but in the converse way, we see that the migration of the bacteria bears no relation to that of the phagocytes.

Egg albumen.—When injected in the raw uncoagulated state this substance was rapidly absorbed; a few phagocytes were withdrawn from the inoculation area by puncture, but none of the bacteria from the intravenous injection were recovered. Albumen coagulated by heat before injection seemed to be practically inert as regards invasion either by phagocytes, or bacteria from the blood-stream.

Fat.—Mutton fat used as a subcutaneous inoculum remained solid in the tissues, and, like the coagulated albumen, remained inert and free both from phagocytes and bacteria.

In a good many of the experiments in which staphylococci were injected intravenously the rabbits became definitely ill before the end of the first week; these when killed almost always showed numerous cortical abscesses in both kidneys. Films and cultures from these abscesses showed the presence of large numbere of staphylococci. In some cases coliform bacilli were also present in small numbers, but failed to grow in culture. I have

no note of an abscess of this type being found in an animal infected only with coli-typhoid bacilli.

DISCUSSION.

How do the organisms find their way into the subcutaneous inoculum? From the fact that the migration in these experiments was limited almost entirely to the coli-typhoid bacteria, it seems that there must be some special characteristic or mechanism which facilitates their passage through the walls of the vascular system, presumably through the capillaries.

So far as we know there is no filtrable form of coliform bacilli which might readily pass out of the capillary walls, and in point of size they are mostly larger than the cocci. The actual motility of the organism might play a part in the case of some of the members of the coli-typhoid group, but the specimens of *B. coli* used in the experiments were not motile, so this mechanism must be ruled out. Mere mechanical lesions of the vessels is negatived in the first place by the fact that in mixed inoculations only one type of organism escapes. Moreover, in some of the experiments the intravenous injection was delayed for 24 hours after the introduction of the gum, so that closure of any ruptured vessels might have time to take place. Another possible way that organisms might migrate is through the medium of phagocytosis. We have seen that phagocytes invade both sterile gum and sterile agar, and the question arises whether they carry the bacilli out with them. We know that the coliform bacillus when inside the phagocyte is in a relatively safe position, as it is protected there from the lysins of the blood-serum. This, then, would be an ideal explanation if it were the true one. As it is the evidence already brought forward in this paper is decidedly opposed to this mode of infection. In addition to the fact that phagocytes may be present (as in experiment 7, with starch, in great numbers) without any bacteria is the fact that where phagocytosis was noted in the fixation abscesses it was never found in the early, but only in the later stages—usually after 24 hours.

The evidence, then, is against phagocytic transportation of the bacteria in these experiments, and we are left to the assumption of a specific spontaneous mode of migation of which no explanation is offered here.

It is possible that all bacteria might resemble the coliform bacilli in readily leaving the blood-stream were it not that the cocci, for instance, are held back by some special mechanism. For instance, O. Thomsen (1920) has shown that the agglutination of organisms in the peritoneal cavity effectually prevents them from obtaining egress to the blood-stream. This, however, seems to throw no light on the experiments of this paper, for the cocci were not agglutinated by the rabbit's serum.

CONCLUSIONS.

(1) Organisms of the coli-typhoid group, when embedded in mucilage, in the subcutaneous tissues of the rabbit or guinea-pig are protected from the defence mechanisms of the host, and may survive for some weeks.

(2) These organisms, when directly inoculated with gum into the sub-

cutaneous tissues, do not produce the intense local reaction with liquefaction of tissues that follows similar local infections with *S. aureus*.

(3) These organisms are able to leave the vascular system without there being any gross mechanical lesion of the vessels. This facility of migration does not appear in the case of *S. aureus* and various other bacteria.

(4) In experimental coli-typhoid septicæmia a subcutaneous fixation abscess of sterile gum tragacanth quickly becomes infected with these bacteria. Staphylococci, on the other hand, rarely gain access to the gum from the blood-stream.

(5) It is suggested that the ready artificial infection of the mucoid bodies by the coli-typhoid bacteria bears a close similarity to the infection of the mucous membranes of the body from the blood-stream, as in the enteric fevers. It is not shown here whether the bodies of this physical character receive the bacilli owing to a selective mechanism, or whether they merely favour their growth on account of the protection they afford them.

(6) The transportation of the bacilli in these experiments apparently was not effected by phagocytes.

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A STUDY OF HÆMOGLOBINOPHILIC BACTERIA BY AGGLUTINATION AND AGGLUTININ ABSORPTION.

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B. influenzæ has been prominently associated with epidemic influenza, but it is well established that the organism may be present in a variety of other pathological conditions and in healthy carriers. The identity or variation of strains from different sources is a matter of considerable interest, since it throws an indirect light on the *rôle* of *B. influenzæ* in the epidemic disease, and is a further step in elucidating the group of hæmoglobinophilic organisms. Pfeiffer recognised that *B. influenzæ* was but one type of this group, and termed other similar organisms "pseudo-influenza bacilli." They, too, have been shown to be pathogenic and have been found in carriers.

For this investigation we have used thirty-eight strains of *B. influenzæ* isolated from hospital patients during a period when no cases of epidemic influenza were seen. They were all Gram-negative bacilli requiring blood-pigment for growth. On plain blood agar they grew in very minute colonies, and caused no hæmolysis after incubation for three days at 37° C. Profuse growth was obtained on chocolate agar (meat infusion agar, neutral to phenolphthalein, with 4 per cent. of glycerine, and the addition of 4 per cent. of citrated blood just before use, while the medium was at boiling-point), and this medium was used as a routine for isolation and subsequent transfer.

Morphologically the strains were not at all alike. The differences were definite, and although one might pick out certain forms as types, this would hardly be justified, since some strains would fall between, and make the variation a gradation rather than a clearly marked grouping. Some were composed of small coccoid bacilli fairly uniform in size and shape, amongst which a few longer forms could be seen. Others showed slender bacilli whose length was 1·5 to 4 times the width, and in some the organisms were mostly much longer, the length six to eight times the width, a few shorter forms

TABLE I.—*Results of Cross-Agglutination.*

Organisms.	Origin.	Limit titre of sera (homologous in heavy type).							
		Wilson.	Kuryk.	Finklestein.	Ford, blood.	A. M.	Gliskmaune.	9.	E.
Wilson	Antrum in rhinitis	**1280**	0	0	80	640	40	0	40
Wilson II	" " "	**640**	0	0	40	0	0	0	0
Arnsden	Pneumonia	1280	0	20	80	640	640	0	20
A. M.	"	1280	0	20	80	**1280**	1280	0	20
Gliskmaune	Subacute bronchitis	1280	0	20	80	640	**2560**	0	20
Leslie, lung	Pneumonia-autopsy	640	0	0	0	320	1280	0	0
Kopf	Chronic bronchitis	320	0	0	80	640	0	0	0
Moore	H.S.C. acute "	640	0	0	80	40	0	0	0
Delamere	Pneumonia	160	0	0	80	1280	2560	0	0
Ford, blood	H.S.C. infected knee	80	0	0	**2560**	20	0	0	0
" knee	" " "	160	0	0	2560	80	160	0	0
Kuryk	Pneumonia	0	**1280**	80	0	20	0	0	0
E	—	320	2560	320	320	80	40	320	**2560**
9	—	0	40	0	0	20	80	**2560**	80
Finklestein	Pneumonia	0	20	**2560**	0	0	0	0	0
Lee	"	320	0	0	20	320	160	0	0
Somers	"	40	0	0	0	160	640	0	0
Wellman	"	0	0	0	0	160	80	0	0
Simmons	"	0	0	0	20	40	160	0	0
Madill	"	0	0	0	40	160	320	0	0
McGreagor	"	0	0	0	20	80	320	0	0
Million	"	160	0	0	0	160	0	0	0
Bellamy	"	0	0	0	160	80	0	0	0
Davis	H.S.C. bronchitis	160	160	0	0	80	640	0	0
Burrows	" "	160	0	0	40	40	0	0	0
Jones	Pneumonia	40	0	20	0	40	0	0	0
Stead	Acute bronchitis	160	0	0	0	80	0	0	0
Gilchrist	Chronic "	0	0	80	20	0	0	0	40
Leslie	Sputum of autopsy case	0	0	0	0	0	0	0	0
Hamilton, mouse	Pneumonia	0	0	0	0	0	0	0	0
" sputum	"	20	0	0	0	0	0	0	0
Sophia	"	20	0	0	0	0	0	0	0
Bradley	"	20	0	0	0	0	0	0	0
Bowen	"	20	0	40	0	0	0	0	0
Morgan	"	0	0	80	0	0	0	0	0
Eisen	Bronchiectasis	0	0	80	0	0	0	0	0
Copeland	H.S.C. meningitis	0	0	0	80	0	0	0	0
Burt	Pneumonia	0	0	20	0	0	20	0	40

For strains marked "H.S.C." we are indebted to Dr. I. H. Erb, who isolated them at the Hospital for Sick Children.
Strains 9 and E were sent to us from the laboratory of Prof. Zinsser, College of Physicians and Surgeons, New York.

being also present. The staining was usually solid, but bipolar forms were seen and irregularity in the centre of longer bacilli.

The origin of the strains is mentioned in Table I. Those from patients with lobar pneumonia, with the exception of two or three, were obtained by culturing the sputum. The exceptions came from the lung at autopsy or from the peritoneal cavity of a white mouse in the course of typing the pneumococcus. The latter was the dominant organism in these cases, but from cultures on chocolate agar it was common to find colonies of *B. influenzæ*, and occasionally in the mouse *B. influenzæ* grew as profusely as the pneumococcus.

TECHNIQUE.

Each strain was isolated by subculturing one colony. Suspensions for agglutinating were made by washing the surface growth from chocolate agar with physiological saline, shaking to break up the clumps, centrifuging out the coarser particles and diluting to a standard density. One-tenth per cent. of formalin was added and the suspensions stored in the ice-chest. The agglutinating sera were obtained from rabbits, and had a titre of 1/1280 or 2560. For absorption of agglutinins killed organisms were left overnight at 37° C. in contact with the serum diluted 1 in 10.

RESULTS.

Table I gives the results of cross-agglutinating thirty-eight strains with eight sera. Dilutions were made from 1 in 20 to 1 in 2560, using seven tubes and doubling the dilution in each. They were incubated in a water-bath at 56° C. for two hours, and the end-point taken as the last tube in which clumping could be seen with the naked eye.

By examination of the figures the strains may be resolved into several groups. The first nine have much in common. The two Ford strains are separate from the others. Kuryk, Finklestein, strains 9 and E, are all different and not related to the others. Strain E is susceptible to agglutination, but has a very narrow antigenic value. The next thirteen are acted upon to a slight extent by two or more sera, but the titres are so much below those for the homologous organisms that these strains fall undetermined outside the former groups. Similarly the last ten strains are an undetermined group, almost untouched by any of the sera.

There was also a difference in the agglutinating value of the sera. Four were quite specific, and did not agglutinate any other strain in a dilution comparable to that of the homologous organism; in fact most strains were not agglutinated at all by them. The other four acted on numerous strains in low dilutions. They have more in common with them than have the four highly specific sera, but although they agglutinated a large portion of the strains to a slight extent, only with the first nine were the titres high enough to suggest a grouping.

These nine strains were investigated by agglutinin absorption. Wilson serum was absorbed by each in turn. The results are given in Table II. In each case a control was made at the same time as the absorbed serum.

TABLE II.—*Results of Absorption Tests using Wilson Serum.*

	Wilson serum absorbed by:									
	Wilson.		Wilson II.		Arnsden.		A. M.		Gliskmaune.	
	Abs. serum.	Cont. serum.	Abs.	Cont.	Abs.	Cont.	Abs.	Cont.	Abs.	Cont.
Wilson	0	640	0	640	160	640	640	1280	160	640
Wilson II	0	640	0	320			640	640	160	640
Arnsden	0	640	40	640	0	640	320	1280	80	640
A. M.	0	640	0	640	40	640	160	640	80	640
Gliskmaune	0	640	0	640	0	640	80	1280	0	640
Leslie, lung	0	160	0	320	0	320	320	1280	0	160
Kopf	0	160	0	160	40	160	320	320	0	160
Moore	0	320	80	320	0	320	160	320	80	320
Delamere	0	160	0	160			160	320		

TABLE II *(cont.)*.

	Wilson serum absorbed by:							
	Leslie, lung.		Kopf.		Moore.		Delamere.	
	Abs. serum.	Cont. serum.	Abs.	Cont.	Abs.	Cont.	Abst.	Cont.
Wilson	160	640	160	640	640	640	320	1280
Wilson II	80	320	160	320	160	320	640	640
Arnsden	80	640	320	640	320	640	80	1280
A. M.	80	640	320	640	160	640	80	640
Gliskmaune	0	640	640	640	320	640	160	1280
Leslie, lung	0	320	160	320	320	320	40	320
Kopf	40	160	0	160	80	160	320	320
Moore	160	320	320	320	0	320	0	320
Delamere	40	160	80	160	0	160	0	320

From these results strains Wilson and Wilson II, isolated from the same person at different times, appear to be identical; the second strain absorbs practically all the agglutinin from the serum of the first, and they react in the same way toward the serum absorbed by other strains. Arnsden is different, and forms a second type. The A. M. organism did not fully absorb, although repeated attempts were made. However, by using the A. M. serum it was found to differ from Arnsden, and is therefore a third type. This is shown in Table III.

TABLE III.—*Results of Absorption Tests using A. M. Serum.*

	Serum A. M. absorbed by:			
	A. M.		Arnsden.	
	Abs.	Cont.	Abs.	Cont.
Arnsden	0	1280	0	1280
Gliskmaune	0	1280	320	1280
A. M.	0	1280	640	1280

That Gliskmaune differs from Arnsden and the previous three is demonstrated by Tables III and I. However, it is identical with Leslie lung, and these two make a fourth type. Similarly Kopf, Moore and Delamere may be isolated. Thus in the first nine strains there are seven serological races.

The two strains from Ford, one from the knee and the other from the blood, are the same, but stand by themselves. So also do Kuryk, E, 9, and Finklestein. Thus in the fifteen strains whose serological character we were able to determine with the sera at hand twelve races were found.

In two instances the identical strains came from the same person. Gliskmaune and Leslie, however, are similar strains from different sources. And it is of interest to note that the two strains obtained from Leslie—one from the lung, the other from the sputum—are not alike.

As to the remaining twenty-three strains, we can only say that with eight sera no grouping was evident, suggesting that, like the first fifteen, they, too, possess a serological individuality.

Our results are in accord with the reports of others who have made similar experiments.

Bell (1920) investigated thirty-seven strains from throats of normal students and influenza cases by agglutination and agglutinin absorption, and found that although identical strains did occur, the group is heterogeneous. He could not make any distinction between strains from normal throats during a non-epidemic period and those from influenza cases. The morphology varied from coccoid to fairly long bacilli, but did not afford a reliable means of grouping and did not correspond to differences in agglutination.

Valentine and Cooper (1919) made similar tests on 171 strains with twenty-five sera, and found no serological relationship.

Povitsky and Denny (1921), working with 185 strains, obtained confirmatory results. Seven from meningitis cases had more in common than the others, four out of the seven belonging to one group.

The conclusions of Coca and Kelly (1921) supported the above findings.

Cohen (1909), comparing strains from meningitis and those from other sources, found that the former differed in agglutination and protection experiments, and produced a fatal septicæmia in rabbits.

CONCLUSIONS.

Viewing our results in the light of those from other laboratories, the following conclusions may be made:

(1) Nearly all strains of *B. influenzæ* possess a serological individuality as determined by agglutination and agglutinin absorption.

(2) Identical strains do occur, but they are not frequent.

(3) The antigenic value of strains varies. Some are extremely narrow. The serum prepared from such strains seldom agglutinated other organisms than the homologous.

(4) The morphological variations are marked, but do not correspond to variations in agglutination.

(5) Two or more serological races may be present in the same patient.

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THE RELATION BETWEEN BILE SALTS AND HÆMOLYSIS IN THE BLOOD-STREAM.

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THE fate of the bile salts in the blood in jaundice is an old and much-discussed question.

The normal amount of bile acids produced in man is about 10 grm. *per diem* (v. Noorden); yet only traces of bile salts are found in normal urine. In the first few days of an attack of jaundice there is evidence that an increased quantity of these substances is absorbed into the blood and excreted in the urine; but afterwards the amount excreted becomes diminished, since bile is excluded from the intestine, and therefore not absorbed. On the other hand, it is stated that in jaundice an increased amount of bile salts enters the blood, and thus produce increased biliary secretion, a vicious circle being established (Wells). In jaundice the presence of bile acids has been demonstrated in the blood (Croftan), and in the thoracic duct (Tapeiner).

It may therefore be taken that, in the first few days of jaundice at least, bile salts are absorbed into the blood-stream in increased amount. The question then arises: Why is there not hæmoglobinuria, and why is so little bile salt excreted in the urine?

Regarding the first part of the question, it is found that hæmoglobinuria is not a symptom in jaundice. A complete list of the conditions in which hæmoglobinuria occurs does not include this disease. It would be expected that, as bile salts are hæmolytic, their injection into the blood-stream would cause hæmoglobinuria; yet this does not appear to happen. That the bile salts could exert their hæmolytic action in the blood-stream seems to be accepted (Wells), and it has been supposed that they are present in insufficient quantity to cause any marked effect. In order to avoid the difficulty, the assumption has been made that part of the bile salts are destroyed in the blood; it has been conclusively shown, however, that all the bile acids absorbed from the intestine are re-secreted in the bile (Stadelmann). In about one-fourth to three-fourths of the cases of jaundice, ranging with the type, no bile salts are found in excess in the urine.

Observations recently published (Ponder) show that, contrary to the usual opinion, bile salts do not exercise their hæmolytic action in the presence of blood serum. The inhibitory action of serum is very great. The presence

of 0·1 c.c. of serum lengthens the time required for the hæmolysis of 1 c.c. of a 5 per cent. suspension of thrice washed human erythrocytes by a 1 in 1000 solution of sodium taurocholate from ½ minute to 7 minutes at 37°. If sodium glycocholate be used as the hæmolysing agent the time is lengthened, in the case of a 1 in 1000 solution acting at 37°, from 2 minutes to 12 minutes. Since these salts do not attain, even in intense jaundice, a concentration of 1 in 1000, or anything approaching it, and since the amount of blood serum present is proportionately much greater, it may be concluded that bile salts exert no hæmolytic action in the blood-stream. This explains the absence of hæmoglobinuria, and also is confirmed by statements that the blood in jaundice, as regards its red cell content, is normal.

It remains to be seen what occurs to the bile salts in the blood-stream. They are known to enter into an adsorption compound with proteins, but this only occurs in the presence of acid. It may be concluded that as the reaction of the blood is not acid, this does not occur; the formation of such a compound would offer considerable difficulty when the facts of re-secretion are considered; the presence of alkali in slight amounts, moreover, favours the inhibitory action of serum. A solution of serum-albumin does not cause this inhibition. It must therefore be concluded that the inhibitory action of serum is not due to the formation of an adsorption compound with bile salts, and that no such combination takes place in the blood-stream.

The bile salts must therefore be considered as being in some state, while in the plasma, other than adsorbed to the proteins—possibly uncombined: their hæmolytic action is, however, inhibited. The problems connected with this inhibition are too complicated for discussion here; the reader is referred to another paper for an account of some of them (Ponder).

Sodium taurocholate and sodium glycocholate have the properties of colloids (Ponder). This, doubtless, explains why they are excreted in such traces only. In the urine it would be expected that, as the reaction is acid, they would be adsorbed by any albumins present. This is actually the case (Wood).

While there seems no doubt that the above statements are true, so far as test-tube experiments are concerned, it is desirable to have animal experiments, in addition, to support such conclusions. A typical animal experiment may be given briefly here:

Rabbit; weight 1785 grm. Chloroform anæsthesia. The jugular vein was exposed and a cannula inserted. The urine was collected from the bladder by a tube. First a sample (1 c.c.) of normal urine was obtained. Into the jugular vein was then injected 2 c.c. of a 1 in 800 solution of sodium taurocholate in saline. After a few minutes the breathing became slightly slower, and the heart-beat slower also. The amount of urine excreted became very much diminished. No hæmoglobinuria appeared. After an interval of about half an hour, when the breathing and heart-beat had become normal, 2 c.c. of a 1 in 80 solution of sodium taurocholate in saline was injected intravenously. This quantity, it may be calculated (Ponder), when evenly distributed throughout the blood of the animal, would form a solution of taurocholate sufficiently powerful, in absence of serum, to hæmolyse 1 per cent. of the total red cells of the animal in seven minutes. A similar slight effect on the respirations and heart-beat occurred. The secretion of urine appeared to cease almost completely. After some time, however, about 1 c.c. was collected; there was no sign of hæmoglobinuria. The animal was killed, some blood drawn off, and the serum separated. The serum was clear, and showed no sign of the presence of hæmoglobin.

It may be observed that the urine collected after injection of the bile salt was more

concentrated than that collected before. Any quantitative estimation of the bile salt excreted in these minute quantities of urine was, of course, impossible. On applying the reaction of Pettenkofer, according to Bertrand (Bertrand and Thomas), there was found to be no bile salts in the first (normal) specimen, and only very slight traces in the urine collected after injection of the bile salts.

These results confirm the conclusions set forth above. The action of the bile salts on the respiratory system and on the heart-beat are described elsewhere (Wells). In the experiments done in connection with this paper they were not nearly so well marked as was expected. The action on the kidney, together with other questions of importance regarding their action, is being further investigated.

CONCLUSIONS.

(1) The bile salts do not cause hæmolysis when in the blood-stream; nor does hæmoglobinuria follow their injection.

(2) This is not due to their forming an adsorption product with the albumins of the serum.

(3) Since they are colloidal in nature, they are with difficulty excreted by the kidney.

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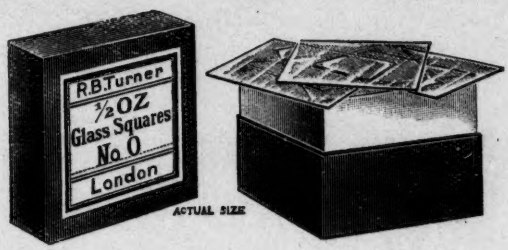

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